Identification of Genus *Vigna* using ITS2 and *matK* as a Two-Locus DNA Barcode

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ABSTRACT DNA barcoding is the use of short DNA sequences of the genome for large scale species identification. The Consortium for the Barcode of Life (CBOL) plant-working group recommended a 2-locus combination as the standard plant barcode. The evolutions of the chloroplast regions combined with nuclear genes are sufficiently rapid to allow discrimination between closely related species. We evaluated the efficacy of the proposed plant barcoding loci, *matK*, along with ITS2 for barcoding the *Vigna* species. To assess the discriminatory ability of barcoding loci for identifying the *Vigna* species, we sampled 52 of the taxonomically best known groups in the genus. Topologies of the phylogenetic trees based on ITS2 and *matK* analyses were similar but a few accessions were placed into distant phylogenetic groups. Neither ITS2 nor *matK* analyses were able to discriminate some closely related *Vigna* species. Thus, we used concatenated data to increase the resolving power of ITS2 and used *matK* as an additional tool for phylogenetic analysis in *Vigna* because characterization of the nucleotide sequences of the *matK* region was easier and more cost-effective than that of the ITS region.

Keywords *Vigna*, rDNA-ITS2, *matK*, phylogenetic analysis, species identification

INTRODUCTION

Legumes (Fabaceae) are the third largest flowering family and are second only to cereals in their importance for human nutrition. Archaeological evidence suggests that the Mediterranean region is the principal center of origin for legumes (Perrino 1988). Legumes are inexpensive, nutrient-dense and high-quality sources of protein that can be substituted for dietary animal protein (Anderson *et al.* 1999). Legumes also produce a high diversity of secondary metabolites that serve as medicinal, nutraceuticals and eco-friendly by-products such as tannins, gums, insecticides, resins, varnish, paints, dyes and biodiesel. There is renewed interest in grain legumes for their use in animal feed. Equally important is the role of legumes in sustainable agriculture: they can fix soil nitrogen via symbiotic nitrogen-fixation and play an important role as a rotation

crop with cereal grain and vegetable crops (Arianoutsou and Thanos 1996; Velazquez *et al.* 2010).

The genus *Vigna* belongs to the Fabaceae family and is divided into seven subgenera, with over 150 species distributed throughout the tropics. Cultivated *Vigna* species are food legumes that have great economic importance throughout the world, especially in developing countries (Smartt 1990). The traditional phenotypic characterization of these species was limited because of weak morphological differentiation. The morphological markers were not quite enough to expose the genetic diversity and do not reflect real genetic relationships. Several DNA marker systems are now commonly used in diversity studies of plants, the most commonly used marker systems are restriction fragment length polymorphism (RFLP) (Soller and Beckmann, 1983), random amplified polymorphic DNA (RAPD) (Williams *et al.* 1990), amplified fragment length polymorphism

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(AFLP) (Vos *et al.* 1995), intersimple sequence repeats (ISSRs) (Zietkiewicz *et al.* 1994) and microsatellites or simple sequence repeats (SSRs) (Becker and Heun 1994).

Recently, DNA barcoding has been used to identify specific regions of the plant genome that can be sequenced routinely in diverse sample sets, resulting in easily comparable data that enable species to be distinguished from one another (Chen *et al.* 2010). Many recent papers have reviewed DNA barcoding in plants (Vijayan and Tsou 2010; Hollingsworth *et al.* 2011). A number of single locus and combined loci have been proposed as barcodes for DNA barcoding (Chase *et al.* 2007; Kress and Erickson 2007). Subsequently, a number of loci in the chloroplast genome have been tested for barcoding and combinations of *rbcL* and *matK* have been proposed as preferred plant barcoding loci (Group *et al.* 2009). Similarly, researchers reported that ITS regions in the nuclear genome also had potential for taxonomic classification and phylogenetic reconstruction (Schultz *et al.* 2005; Yao *et al.* 2010; Bellemain *et al.* 2010). Because of sufficient variation in the primary sequences and secondary structures of the ITS2 region, a combination with this locus is considered efficient for plant barcoding. Hence, the China Plant BOL Group proposed the addition of nuclear ITS2 to the *matK* + *rbcL* combination as a plant barcode (Li *et al.* 2011). In the present study, we therefore evaluated the use of a nuclear region ITS2 along with a chloroplast region *matK* for the discrimination of *Vigna* species.

MATERIALS AND METHODS

Plant Material and DNA extraction

Seeds of genebank accessions (a total of 149 accessions) belonging to 52 species of *Vigna* were provided by the Genetic resource center of the National Academy of Agricultural Science, Rural Development Administration, Republic of Korea (Table S1). To determine which of a subset of proposed barcoding loci are necessary to resolve *Vigna* species, we sampled a total of 149 accessions that contained two to three individual accessions. Seeds were germinated and leaf tissue was harvested from 3 week old seedlings in the greenhouse. Total DNA was extracted

using the DNeasy® Plant Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Fresh leaf tissue from each accession was used for each extraction and was ground well in liquid nitrogen. DNA was resuspended in 100 µL water, diluted to 10 ng/µL and stored at either -20°C or -80°C. Genomic DNA was quantified using a Nanodrop/UVS-99 instrument (ACTGene, USA), and the A260/A280 nm ratio was determined. DNA quality was confirmed on a 0.8% agarose gel.

PCR amplification and Sequencing

The sequences of universal primers for ITS2 and *matK* regions as well as general PCR reaction conditions were obtained from previous studies (Chen *et al.* 2010). Amplification reactions were carried out in a total volume of 20 µL containing 1× PCR buffer, 0.1 mM primers, 0.2 mM of each dNTP, 1 U Taq DNA polymerase and 200 ng template DNA. Approximately 1 µg DNA was used to generate a DNA library for use with the Genome Sequencer GSFLX Titanium System (Roche, 454 Life Science, Branford, CT, USA). The DNA fragment ends were polished (blunted), and two short adapters were ligated to both ends according to standard procedures (Moe *et al.* 2011). Finally, the quality of this single stranded template DNA library was assessed using a 2100 BioAnalyzer (Agilent, Waldbronn, Germany). The library was quantified to determine the optimal amount needed as input for emulsion-based clonal amplification. Multiplex identifiers were used to specifically tag unique samples in a GS FLX Titanium sequencing run, which were recognized by the GS data analysis software after the sequencing run and provided high confidence for assigning individual sequencing reads to the correct sample.

Phylogenetic analysis

Sequences of ITS2 and *matK* regions were manually edited with MEGA6 (Tamura *et al.* 2013) and the sequences were aligned using the "ClustalW" option. Manual adjustment in the alignment of the nucleotide sequences for the ITS2 region was made to improve alignment. Aligned ITS2 and *matK* sequences were evaluated with bootstrap analysis (1000 replicates) (Felsenstein 1985) and Kimura-2-parameter distances (Kimura 1980) were calculated to construct a

neighbor-joining (NJ) dendrogram using the MEGA6 (Tamura *et al.* 2013).

RESULTS

We employed molecular markers to test the discriminatory power of each DNA barcode region. Genomic DNA extracted from *Vigna* samples and typical PCR amplicons obtained for the DNA barcodes used here are shown in Table 1. Amplification of ITS2 and *matK* DNA barcodes for all *Vigna* populations analyzed here yielded alignments of 413 and 469 bp, and the resulting concatenated sequence length of ITS2 and *matK* was 882 bp. We obtained 149 ITS2 and 149 *matK* sequences from 52 *Vigna* species, resulting in 298 new sequences. All 52 individuals in each population were found to be 100% identical at the DNA level. All positions containing gaps and missing data were eliminated. There were a total of 277 positions in the final dataset.

A total of 298 sequences were analyzed from 52 *Vigna* species in this study. Sequence analysis revealed the average nucleotide frequencies as A = 23.3 ± 1.50 %, T = 25.4 ± 4.70 %, G = 28.5 ± 1.60 %, C = 22.8 ± 3.40 %. The phylogenetic utility of ITS2 and *matK* regions is shown in Table 1. The number of segregating sites (S) = 277 and the nucleotide diversity (π) = 0.196706. Tajima's statistics (D) = 0.329429 and it was not significantly ($p < 0.01$) different among sequences. The Kimura 2 Parameter (K2P) genetic distance in *Vigna* species is given in Table S2. The overall K2P distance between the 52 species was 0.240.

An un-rooted phylogenetic tree constructed by NJ based on ITS2 sequence data is presented in Figure S1. The phylogenetic analyses based on the ITS2 region generally discriminated the *Vigna* species. The 149 accessions analyzed in this study were placed into six Groups (I–VI)

supported by high bootstrap values. Four *Vigna* species analyzed (*V. reticulata*, *V. frutescens*, *V. heterophylla* and *V. vexillata* varieties) were placed in Group I. Species and subspecies of *V. friesiorum* and *V. membranacea* were placed in Group II. *V. schimperi* formed a distant Group III. Five *Vigna* species (*V. unguiculata*, *V. radiata*, *V. lobatifolia*, *V. angivensis*, and *V. vexillata*) and their subspecies were placed in Group IV. Six *Vigna* species (*V. hosei*, *V. racemose*, *V. fischeri*, *V. oblongifolia*, *V. marina*, and *V. luteola*) and their varieties formed Group V. The remaining *Vigna* species and their subspecies were grouped together with a 66% bootstrap value as Group VI. Within Group VI, there were three subclades supported by high bootstrap values.

Similarly, phylogenetic analyses based on the nucleotide sequences of *matK* region generally discriminated the *Vigna* species (Fig. S2). The 149 accessions analyzed in this study were placed into distant clades if they were not supported by high bootstrap values (Fig. S2). The *matK* sequences separated most of the species in the species *Vigna* from each other but some closely related species could not be discriminated.

A neighbor-joining (NJ) phylogenetic tree was developed based on the concatenated ITS2 and *matK* sequences. The topologies of the phylogenetic NJ tree were similar (Fig. 1). Bootstrap values for all nodes ranged between 59 to 100%, with one short branch within a clade retaining a 57% bootstrap value in the NJ tree. Similar to ITS2, the concatenated sequences based NJ tree separated the 52 analyzed *Vigna* species into six groups (I–VI): (i) group I contained *V. reticulata*, *V. frutescens*, and *V. heterophylla* and *V. vexillata* varieties, (ii) group II included species and subspecies of *V. friesiorum* and *V. membranacea*, (iii) group III comprised *V. schimperi* as a distant clade, (iv) group IV contained five *Vigna* species (*V. unguiculata*, *V. lobatifolia*, *V. angivensis*, and *V. vexillata*) with one short

Table 1. Genetic diversity of barcoding markers and combinations used in this study.

Marker combination	Individuals (n)	Number of species	Aligned length	Variable characters	Number of segregating sites	Nucleotide diversity (π)
ITS2	149	52	413	29	200	0.137552
<i>matK</i>	149	52	469	21	76	0.016086
ITS2 + <i>matK</i>	149	52	882	33	277	0.196706

Fig. 1. Phylogenetic analysis of 52 *Vigna* species based on the nucleotide sequences of the ITS2 and *matK* regions. The NJ tree was developed using the Kimura 2-parameter method with nucleotide sequences of the ITS2 and *matK* regions. Numbers next to the branches are the bootstrap test values.

branch (*V. radiate*) within the group, and their subspecies, (v) group V included six *Vigna* species (*V. hosei*, *V. racemose*, *V. fischeri*, *V. oblongifolia*, *V. marina*, and *V. luteola*), and (vi) group VI included the remaining 19 *Vigna* species and subspecies, varieties of *V. trinervia*, *V. reflex-pilosa*, *V. glabrescens*, *V. hirtella*, *V. nakashimae*, *V. riukiensis*, *V. mungo*, *V. tenuicaulis*, *V. aconitifolia*, *V. angularis*, *V. nepalensis*, *V. stipulacea*, *V. trilobata*, *V. peduncularis*, *V. Caracalla*, *V. linearis*, *V. lasiocarpa*, *V. longifolia*, and *V. frutescens* subspecies. Within Group VI, the NJ tree based on concatenated ITS2 and *matK* sequences successfully discriminated 19 *Vigna* species. Moreover, the *matK* sequences improved the bootstrap values for all nodes in the phylogenetic tree (Fig. 1). Although this barcoding region successfully discriminated all 52 *Vigna* species, closely related species were not discriminated.

DISCUSSION

DNA barcoding approaches using more samples (species/accessions) provides better insights into species relationships as well as more precise estimation of intraspecific heterogeneity. Morphological, biochemical, and molecular marker approaches have been used to infer the genetic relationships within and among *Vigna* species (Maxted *et al.* 2004). Various studies have been published investigating the phylogenetic relationships among species based on cpDNA (Javadi *et al.* 2011), rDNA (Vijaykumar *et al.* 2010), AFLP (Tomooka *et al.* 2002), RAPD analysis (Mimura *et al.* 2000) and biochemical markers (Yasuda and Yamaguchi 1996). Similarly, FISH mapping of rDNA in *Vigna* species was also previously conducted (She *et al.* 2015). However, none of these have efficiently resolved the taxonomic problems.

The internal transcribed spacer (ITS) regions of 45S rDNA have been used extensively to determine phylogenetic relationships at the interspecific or intraspecific level because of their relatively high rate of mutation (Alvarez and Wendel 2003). Previously, the ITS region was proposed for phylogenetic analysis in plant species (Chen *et al.* 2010; Gao *et al.* 2010). Recently, nucleotide sequences of some

DNA regions in the chloroplast genome (*matK*, *rpoC1*, *rpoB*, *trnH-PsbA*, *rbcL*, *atpF-atpH*, *psbK-psbI*) and their combinations were tested for barcoding plant species (Starr *et al.* 2009). Among these DNA regions, *matK* and *rbcL* were accepted as a 2-locus DNA barcode by the CBOL Plant Working Group (Group *et al.* 2009). In this study, potential use of ITS2 nucleotide sequences combined with *matK* was investigated as a two-locus DNA barcode for discrimination of the *Vigna* species.

In the present study, ITS2 and *matK* sequences were found to be easy to amplify with polymerase chain reaction (PCR). A total of 149 accessions were analyzed, from which 298 sequences (all > 400 bp) belonging to 52 *Vigna* species were ultimately obtained. Nucleotide diversity rate tests were performed for the aligned ITS2 and *matK* sequences with all pairwise combinations in the genus *Vigna*. Genetic divergence, represented by the average genetic distance of all pairwise comparisons, was 0.137 for the ITS2 region and 0.016 for the *matK* region (Table 1). Genetic distance estimated with the Kimura 2- parameter method (Kimura 1980) among taxa calculated from complete sequence data ranged from 0.000 to 0.701 (Table S2). This is a general problem for large-scale barcoding, as demonstrated by (Bergsten *et al.* 2012), who concluded that the genetic distance to the closest heterospecific region decreases with increasing geographical sampling scale, linked to the larger intraspecific variance when more populations are incorporated. Thus, we included more samples and species per genus, in order to find the genetic distance between populations.

Species resolution of both DNA regions was similar because the topologies of ITS2 and *matK* trees were similar. Both DNA regions were generally separated in *Vigna*. However, several species could not be discriminated from each other by either ITS2 or *matK* regions. It was reported that the ITS region requires cloning before sequencing because of allelic polymorphisms, pseudogenes, and paralogous copies of the ITS region in plant species (Bailey *et al.* 2003; King and Roalson 2008). However, there was no allelic polymorphism or insertion/deletion in the *matK* region within the chloroplast genome of the *Vigna* species. Therefore, we were able to efficiently amplify and sequence-characterize the *matK* region without cloning.

The NJ method was originally employed in this study for species identification, and all accessions were allocated into different groups without polyphyly/paraphyly in the NJ tree (Fig. 1). Phylogenetic relationships among *Vigna* species based on ITS2 analyses were in agreement with previous studies using the ITS region (Vijaykumar *et al.* 2010). The genetic diversity and phylogenetic relationships among species in the *Vigna* subgenus *Ceratotropis* were also investigated using sequence data from the ribosomal DNA ITS and *atpB-rbcL* intergenic spacer of chloroplast DNA regions (Doi *et al.* 2002). Similarly in the present study, the ITS2 and *matK* sequences proved efficient for discriminating *Vigna* species. However, in the present study we found that the *V. vexillata* and their varieties were present in different clade; the accession need to be tested with more samples along with multiple locus. The sequence data obtained for ITS2 and *matK* were used to discriminate the species used. The dendrogram clearly indicated diversity among the *Vigna* accessions. Thus our results suggest that ITS2 and *matK* could make a significant contribution to resolving phylogeny at multiple taxonomic levels from generic relationships within *Vigna* species.

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